Induction of SHIP deficiency prior to allogeneic bone marrow transplant enhances engraftment and survival

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Abstract

Graft-Versus-Host Disease (GVHD) is the leading cause of treatment related death in allogeneic bone marrow transplantation (BMT).[1] Immunosuppressive strategies to control GVHD are only partially effective and often lead to life-threatening infections.[2] We previously showed that engraftment of MHC mismatched BM is enhanced and GVHD abrogated in recipients with germline SHIP-deficiency.[3-5] Here we show that induction of SHIP deficiency prior to allogeneic BMT enhances engraftment and abrogates GVHD such that survival is comparable to that of autologous BMT recipients. These findings indicate strategies that target SHIP might increase the efficacy and utility of allogeneic BMT and thereby provide a curative therapy for a wide spectrum of human diseases.

We previously found that the SH2-containing Inositol Phosphatase (SHIP) is critical for maintenance of NK receptor repertoire (NKRR) diversity.[3, 5] Disruptions of the NKRR caused by germline SHIP deficiency enhance engraftment of BM from donors with complete MHC mismatches.[3, 5] $SHIP^{-/-}$ HSC also exhibit spontaneous mobilization and reduced BM retention which may also facilitate engraftment of donor HSC in SHIP-deficient BMT recipients.[6] Furthermore, acute and chronic GVHD are reduced in $SHIP^{-/-}$ BMT recipients[3] due to a profound expansion of myeloid immunoregulatory cells in secondary lymphoid tissues.[4] Although this confluence of genetic abnormalities compromises the viability of germline $SHIP^{-/-}$ mice,[4, 7] it also led us to propose that induction of a short period of SHIP deficiency just prior to allogeneic BMT might enhance engraftment and survival even in settings where donor and host have a complete MHC mismatch.[3, 4] In order to test this hypothesis we developed a genetic model in which SHIP deficiency can be induced in adult recipients using an inducible Cre transgene[8] on a $SHIP^{flox/flox}$ background.

To test this hypothesis we first established cohorts of $MxCreSHIP^{flox/flox}$ and $SHIP^{flox/flox}$ mice. Employing a poly(I/C) adminstration regimen utilized by Mikkola et al in $SCL^{flox/flox}$ mice[9] we typically find that three consecutive injections of poly(I/C) are sufficient to render most adult $MxCreSHIP^{flox/flox}$ mice fully SHIP deficient. However, two poly(I/C) injections leads to induction of partial SHIP deficiency in the majority of $MxCreSHIP^{flox/flox}$ mice as SHIP expression is reduced, but detectable in the overwhelming majority of $MxCreSHIP^{flox/flox}$ receiving two poly(I/C) injections. Since

full and prolonged SHIP deficiency poses a significant threat to viability, we chose to pursue the latter strategy to induce SHIP-deficiency. As expected MxCreSHIP$^{flox/flox}$ mice had detectable, but reduced SHIP expression in their peripheral blood mononuclear cells (PBMC) 6 days after the initial poly(I/C) injection while two mice had no detectable SHIP expression (Fig. 1a). The former were considered to be partially SHIP-deficient, while the latter were considered fully SHIP-deficient. Seven days after the induction of SHIP deficiency we initiated a fully mismatched allogeneic BMT procedure in both the poly(I/C)-treated MxCreSHIP$^{flox/flox}$ cohort and identically treated SHIP$^{flox/flox}$ cohort. This involved myeloablation by irradiation from a ^{137}Cs source and transplantation with 15X10^6 whole BM cells and 15X10^6 splenocytes from a BALB/C (H2d) donor. The MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ transplant recipients are on a C57BL6/J (H2b) background and thus are completely mismatched to the donors at all major MHC loci. In parallel we performed an autologous BMT procedure on a cohort of C57BL6/J (H2b) mice. Survival was monitored in all three BMT cohorts for 112 days (16 weeks) post-transplant. Long-term survival at this point post-transplant in the autologous BMT cohort was 100%, 94% in the MxCreSHIP$^{flox/flox}$ (SHIP-deficient) cohort and 57% in the SHIP$^{flox/flox}$ (SHIP competent) cohort (Fig. 1b). Comparison of survival in the SHIP-deficient and SHIP-competent cohorts by the Kaplan-Meier log-rank test indicated survival was significantly enhanced by induction of SHIP deficiency prior to allogeneic BMT ($p=0.040$). As expected survival of the SHIP-competent cohort receiving allogeneic BMT was also significantly reduced relative to the autologous BMT cohort ($p=0.001$). However, the Kaplan-Meier log-rank test indicates there was comparable

long-term survival in the SHIP-deficient allogeneic BMT and the autologous BMT cohorts ($p=0.232$) (Fig. 1b). These findings are consistent with our previous allogeneic BMT studies in germline SHIP$^{-/-}$ mice,[3-5] but importantly they show that induction of SHIP deficiency in the adult just prior to a fully-mismatched, T cell replete transplant can provide protective benefit without significant toxicity.

In addition to survival, we also monitored weight and clinical measures of GVHD post-transplant in the SHIP-deficient and SHIP-competent cohorts (Fig. 2). Prolonged SHIP-deficiency causes weight loss and wasting in germline SHIP-deficient mice due to a macrophage-mediated consolidation of the lungs.[7, 10] However, induction of SHIP-deficiency for one week did not cause a significant drop in the weight of the SHIP-deficient cohort relative to the identically treated SHIP-competent cohort ($p=0.39$). Despite starting at a comparable weight immediately prior to BMT, the weight of the SHIP-deficient cohort rebounded and increased significantly relative to that of the SHIP-competent cohort during the acute recovery phase of transplant ($p<0.01$) (Fig. 2a). Based on a scoring system that assesses different features of GVHD, including skin integrity, fur texture, posture and activity[11], the SHIP-deficient cohort exhibited fewer or reduced manifestations of GVHD during the acute recovery period. This disparity was most apparent 3-4 weeks post-transplant (Fig. 2b). Histopathological analysis of GVHD in key target organs (skin, liver and the gastrointestinal tract) confirmed the presence of GVHD in all mice that succumbed post-transplant (Fig. 3).

To confirm that mice were engrafted with donor BM, we analyzed multi-lineage repopulation 8 weeks post transplant in the surviving mice. Flow cytometric analysis of peripheral blood mononuclear cells (PBMC) was used to determine donor BM contribution to the T, B and myeloid lineages (Fig. 4). This analysis showed that all mice in both cohorts had significant donor repopulation in all three lineages with no significant difference in repopulation observed for the T lymphoid or myeloid lineages or for global hematopoietic repopulation ($CD45^+$ cells) (Fig. 4a,c,d). However, B lymphoid repopulation by donor BM was significantly higher in the SHIP-deficient allogeneic BMT cohort relative to the SHIP-competent allogeneic BMT cohort (Fig. 4b). Humoral immune responses to T-dependent antigens are severely compromised following allogeneic BMT[12]. Indeed, recovery of antibody responses to key pathogens is an important milestone indicative of successful recovery from allogeneic BMT[13, 14]. The enhanced B lymphoid recovery that we observe in mice rendered SHIP-deficient prior to transplant may be a contributing factor in their increased survival.

These findings demonstrate that induction of SHIP-deficiency in the adult allogeneic BMT recipient enhances both the quality and duration of their post-transplant survival. Indeed, survival is comparable to that of mice that underwent autologous BMT. In addition, recovery of B lymphopoiesis is also enhanced. These findings suggest that pharmaceutical approaches to target the expression (e.g., RNAi) or activity (e.g. low molecular weight inhibitors) of SHIP might potentially be deployed to reduce the incidence and severity of GVHD without the necessity for broadly acting

immunsuppressive regimens. Although full and prolonged SHIP-deficiency clearly has deleterious consequences,[7] our findings indicate transplant success can be achieved with induced SHIP-deficiency even when a state of partial SHIP deficiency exists prior to transplant. Importantly this protection can be achieved in a transplant regimen where donor and recipient are completely mismatched and despite the BM graft being supplemented with substantial numbers of donor peripheral T cells. Success with induced SHIP-deficiency in a BMT regimen that includes this donor leukocyte supplement suggests this strategy might also be used to facilitate graft-versus tumor effects in allogeneic BMT protocols for malignancies. In addition to increasing the efficacy of allogeneic BMT with histocompatible donors, our findings indicate induction of SHIP deficiency might also be explored with incompatible donors. This latter situation would substantially increase the utility of a clinical procedure that has the capacity to cure genetic, autoimmune and malignant diseases.

METHODS

Animals. Mice with germline transmission of $SHIP^{flox}$ allele were previously created in our lab[3] and were maintained by intercrossing $SHIP^{flox/flox}$ mice (F10 to the C57BL/6J background). $MxCreSHIP^{flox/flox}$ and $SHIP^{flox/flox}$ littermates were generated for the BMT study by intercrossing $MxCreSHIP^{flox/+}$ and $SHIP^{flox/flox}$ mice. All studies were performed in accordance with the guidelines and approval of the Institutional Animal Certification and Use Committee (IACUC) at the University of South Florida.

Antibody staining and flow cytometry. CD16/32 was co-incubated with the samples to block Fc receptor binding. Primary antibodies included: anti-H2d, anti-H2b, anti-B220, anti-CD3, anti-Mac1, and anti-Gr1 and were purchased from BD Pharmingen (San Jose, CA). Samples were acquired on a FACS-Calibur and analyzed using FlowJo6.6 software.

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Figure Legends

Fig. 1. Induction of SHIP-deficiency in the host enables long-term survival in complete MHC mismatched BM transplant. (a-c) Western blot analysis of SHIP expression in PBMC of representative poly(I/C)-treated $SHIP^{flox/flox}$ (a) and $MXCreSHIP^{flox/flox}$ (b,c) mice prior to BMT. The mouse in (b) is representative of the majority of the $MXCreSHIP^{flox/flox}$ cohort that had partial deficiency prior to transplant while (c) indicates one of the two animals in the cohort that had no detectable SHIP expression prior to BMT. All $SHIP^{flox/flox}$ and $MXCreSHIP^{flox/flox}$ mice were injected with 625μg polyI/C on days 1 and 4. On day 7 mice were bled and PBMC probed for SHIP and β-actin. On day 8 the mice received 1000 Rads from ^{137}Cs source as a split dose (600+400) and the mice were then transplanted with $15x10^6$ BM cells and $15x10^6$ splenocytes from BALB/C (H2d) donors. All recipients were on a C57BL6/J (H2b) background. Mice we maintained on autoclaved bedding, water and chow in microisolator cages for the duration of the study. (d) Kaplan-Meier step-function of survival in the $MXCreSHIP^{flox/flox}$ (SHIP-deficient) (n=15) and $SHIP^{flox/flox}$ (SHIP-competent) (n=14) cohorts following allogeneic BMT. In parallel we also performed an autologous BMT on a cohort of C57BL6/J using $10X10^6$ WBM cells. (p=0.001, $SHIP^{flox/flox}$ allogeneic BMT vs. autologous BMT cohort; p=0.040, $MxCreSHIP^{flox/flox}$ allogeneic BMT vs. $SHIP^{flox/flox}$ allogeneic BMT cohort; p=0.232, $MxCreSHIP^{flox/flox}$ allogeneic BMT vs. autologous BMT cohort)

Fig. 2. SHIP-deficient allogeneic BMT recipients exhibit reduced GVHD symptoms. (a) Analysis of weight in the SHIP-deficient and SHIP-competent cohorts post-transplant. (*p<0.01) (b) Average scores for rating of clinical manifestations of GVHD in which posture, skin integrity, fur texture and activity were rated on a scale of 0-2 (0, no evidence of disease; 2, clear evidence of disease) as described by Cooke *et al.*[11] All scores represent the average of individual scores from three independent investigators. (*, p<0.05)

Figure 3. Histopathological evidence of GVHD in mice that succumbed post-transplant. Formalin fixed tissue sections from all mice that succumbed in the BMT study described above were analyzed for evidence of GVHD in key target organs (skin, liver and small intestine) in a blinded fashion by a veterinary pathologist (RWE). All mice that succumbed showed histopathological evidence of GVHD. Examples of observed histopathology referrable to GVHD in the skin (a), liver (c) and small intestine (e) are shown, and compared to the skin (b), liver (d) and small intestine (f) of healthy C57BL6/J mice. (a) Skin of mice with GVHD showed pyknosis and vacuolation of epidermal cells in the basal layer and a mild lymphocytic infiltrate in the dermis, compared to unaffected skin of a C57BL6/J control (b). Liver of mice with GVHD (c) showed bile duct destruction and regeneration and a moderate, primarily lymphocytic infiltrate in portal areas with attendant destruction of hepatic parenchyma (between arrowheads & within inset), compared to the unaffected liver and portal triads (arrowhead, inset) of a C57BL6/J control (d). Small intestine of mice with GVHD (e)

showed glandular destruction, moderate lymphocytic infiltrate, and focal loss of mucosa, compared to the unaffected intestine of a C57BL/6J control (f) (hematoxylin & eosin 200X, insets 400X).

Fig. 4. Multi-lineage engraftment by donor BM and enhanced B cell repopulation in SHIP-deficient allogeneic BMT recipients. (a) Global donor hematopoietic repopulation in SHIP-deficient ($MXCre^+$) and SHIP-competent ($MXCre^+$) transplant recipients. (b) B lymphoid repopulation in the same mice as in (a). (c) T lymphoid repopulation in the same mice as in (a). (d) Myeloid repopulation in the same mice as in (a). Donor repopulation was assessed at two months post-transplant by flow cytometric analysis of PBMC as previously described.[3] Donor and host repopulation in each lineage were assessed with H2d and H2b specific antibodies and appropriate lineage markers (B220, B cells; CD3, T cells, Mac1+Gr1, myeloid). The indicated p-values are for comparison of repopulation in the SHIP-deficient and SHIP-competent allogeneic BMT cohorts.

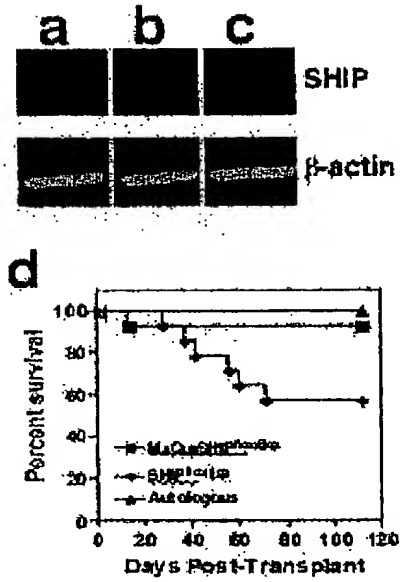


Fig. 1 Paraiso et al

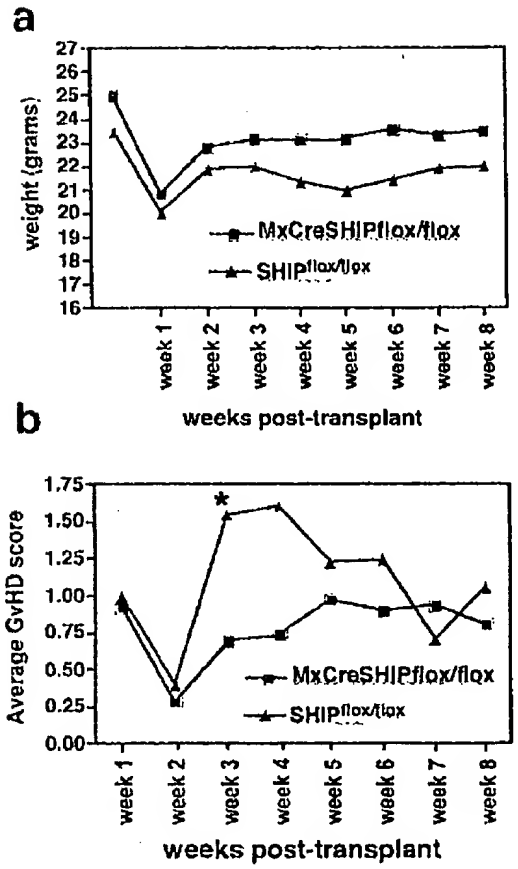


Fig 2 Paraiso et al

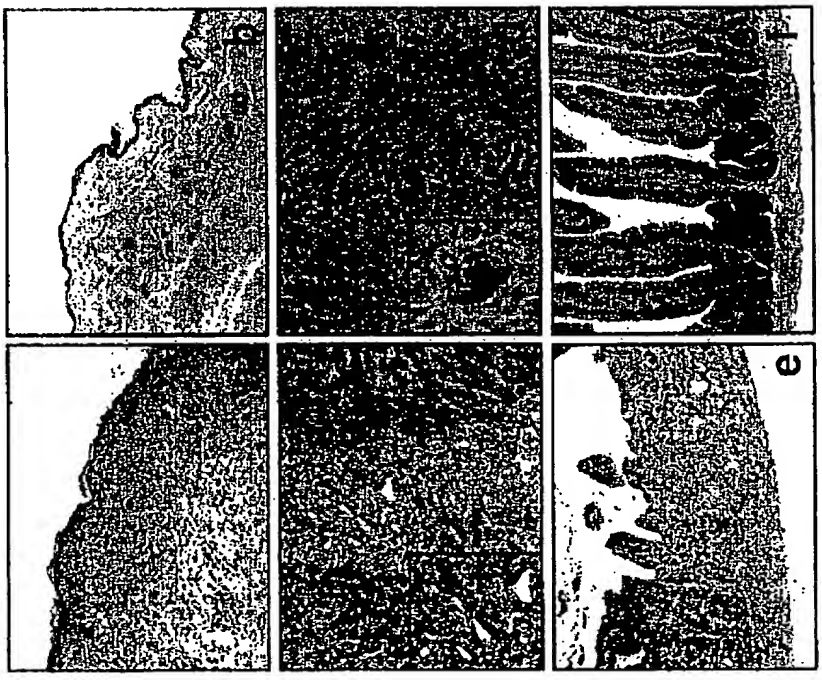
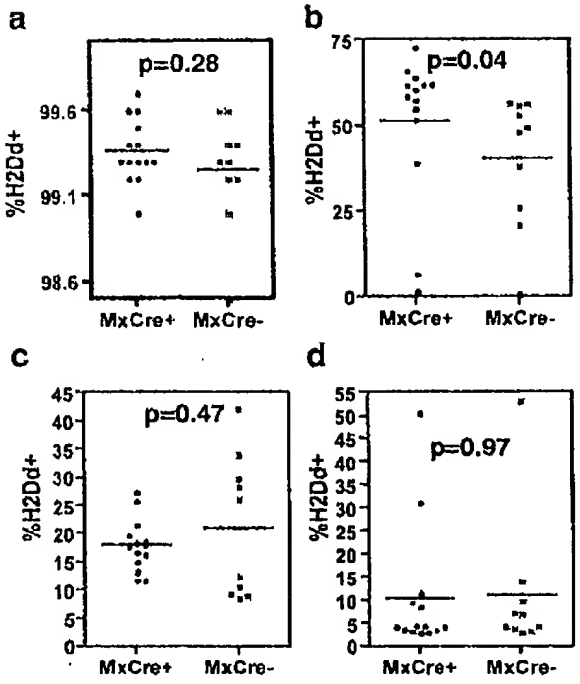


Fig 3 Paraiso et al



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